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Muscular diacylglycerol metabolism and insulin resistance

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Abstract

Failure of insulin to elicit an increase in glucose uptake and metabolism in target tissues such as skeletal muscle is a major characteristic of non-insulin dependent type 2 diabetes mellitus. A strong correlation between intramyocellular triacylglycerol concentrations and the severity of insulin resistance has been found and led to the assumption that lipid oversupply to skeletal muscle contributes to reduced insulin action. However, the molecular mechanism that links intramyocellular lipid content with the generation of muscle insulin resistance is still unclear. It appears unlikely that the neutral lipid metabolite triacylglycerol directly impairs insulin action. Hence it is believed that intermediates in fatty acid metabolism, such as fatty acyl-CoA, ceramides or diacylglycerol (DAG) link fat deposition in the muscle to compromised insulin signaling. DAG is identified as a potential mediator of lipid-induced insulin resistance, as increased DAG levels are associated with protein kinase C activation and a reduction in both insulin-stimulated IRS-1 tyrosine phosphorylation and PI3 kinase activity.

As DAG is an intermediate in the synthesis of triacylglycerol from fatty acids and glycerol, its level can be lowered by either improving the oxidation of cellular fatty acids or by accelerating the incorporation of fatty acids into triacylglycerol.

This review discusses the evidence that implicates DAG being central in the development of muscular insulin resistance. Furthermore, we will discuss if and how modulation of skeletal muscle DAG levels could function as a possible therapeutic target for the treatment of type 2 diabetes mellitus. © 2007 Elsevier Inc. All rights reserved.

Keywords: Diacylglycerol; Protein kinase C; Skeletal muscle insulin resistance; Lipid accumulation

1. Introduction

During the past decades, the prevalence of obesity in children and adults has increased enormously. This is a major health issue at the moment, because the incidence rate of obesity is currently reaching epidemic proportions and given the current trend there are no signs that it will decrease. Obesity is a condition characterized by excess body fat and is defined with a body mass index (in kg/m^2) ≥ 30 . Currently, 30% of American adults are classified as obese and 1 out of every 6 children is overweight according to the National Health and Nutrition Examination Survey [1]. Obesity is clearly recognized as a deleterious condition and predisposes individuals to a range of health complications including: insulin resistance [2], type 2 diabetes [2], hypertension, cardiac failure, and atherosclerosis, which accounts for a high percentage of the current health care costs [3–6].

Although the association between obesity and insulin resistance is clearly shown in epidemiologic literature, the pathophysiology of obesity and insulin resistance needs to be further

Abbreviations: DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty acids; IMTG, intramyocellular triacylglycerol; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PIP₂, phosphatidylinositol 3,4 bis-phosphate; PIP₃, phosphatidylinositol 3,4,5 triphosphate; PDK1, phosphoinositide-dependent kinase 1; TZD, Thiazolidinediones; PPAR γ , peroxisome proliferator receptor γ ; LCACoA, long-chain acyl-CoA; SPT, serine palmitoyltransferase; TNF α , tumor necrosis factor α ; PLD, phospholipase D; PC, phosphatidylcholine; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidate acyltransferase; PA, phosphatidic acid; PPH-1, phosphatidic acid phosphohydrolase; DGAT, diacylglycerol acyltransferase; HSL, hormone sensitive lipase; ATGL, AdiposeTriGlycerideLipase; PS, phosphatidyl serine; cPKC, classical protein kinase C; nPKC, novel protein kinase C; aPKC, atypical protein kinase C; SCD-1, stearoyl-CoA desaturase; CPT-1, carnitine palmitoyl transferase 1; MGAT, acyl-coA: monoacylglycerol acyltransferase.

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elucidated. Insulin resistance is characterized by the reduced ability of the pancreatic hormone insulin to promote peripheral glucose disposal and suppress hepatic glucose output. Skeletal muscle is considered as one of the primary tissues in glucose homeostasis, because it accounts for 75–80% of whole body insulin-stimulated glucose uptake [7,8]. Therefore, skeletal muscle insulin resistance is a major determinant of hyperglycemia and type 2 diabetes mellitus. Skeletal muscle insulin resistance has been associated with the accumulation of total body fat [9]. However, an even stronger association has been shown between intramyocellular fat storage and insulin resistance in animals [10,11] and humans [12,13]. This suggests that aberrant storage of lipids or lipid intermediates in skeletal muscle contributes to the development of insulin resistance [14].

The aim of this review is to discuss the association between skeletal muscle insulin resistance and ectopic lipid accumulation. First we will discuss the normal insulin signalling cascade and the interference of ectopic lipids. Next, the role of the different lipid intermediates in the development of muscular insulin resistance will be highlighted, with a focus on DAG.

2. Insulin signalling pathways promoting glucose uptake

Insulin promotes the uptake of glucose from the plasma into skeletal muscle (Fig. 1). The insulin receptor (IR) is an $\alpha_2\beta_2$ heterodimeric transmembrane protein that possesses intrinsic protein tyrosine kinase activity. After binding of insulin to the extracellular domain of the α subunit, the receptor undergoes conformational changes that result in autophosphorylation of specific tyrosine residues in the cytoplasmic domain of the β subunit. Autophosphorylation of tyrosine residues stimulates the catalytic activity of receptor tyrosine kinase and creates recruitment sites for insulin receptor substrate proteins, such as insulin receptor substrate-1 (IRS-1) [15–17]. Phosphatidyl inositol 3-

kinase (PI3K) is a target of IRS proteins that phosphorylates phosphatidyl inositol 4,5-bisphosphate (PIP₂) to form phosphatidyl inositol 3,4,5-trisphosphate (PIP₃), which in turn activates phosphatidyl inositol-dependent kinase1 (PDK1). This Ser/Thr kinase then activates protein kinase B (PKB or Akt) and the atypical protein kinase C (PKC) isozymes PKC ζ and PKC ι/λ [18]. Both Akt and the atypical PKCs have been implicated in the translocation of the glucose transporter 4 (GLUT4) from the cytosol to the plasma membrane, which mediates glucose uptake.

3. Lipid-induced insulin resistance in skeletal muscle

In the past 15 years, evidence has accumulated that increased plasma free fatty acid (FFA) levels are associated with insulin resistance [19–21]. Indeed, most insulin-resistant obese people have elevated plasma FFA levels and acute elevations of plasma FFA in diabetic or non-diabetic individuals induces insulin resistance in a dose-dependent way [22–24]. Moreover, lowering plasma FFA levels by Acipimox a derivate of nicotinic acid, which inhibits lipolysis, normalized insulin sensitivity in obese and non-diabetic subjects, and significantly improved it in obese diabetic patients [25]. In addition, treatment of high-fat fed mice with acipimox lowered FFAs in the plasma and significantly improved insulin sensitivity [26].

Several studies have aimed to elucidate the role of elevated FFA levels in the onset of insulin resistance. Diet intervention studies in rodents showed that insulin resistance develops after a few weeks of high-fat feeding and is associated with the accumulation of intramuscular triacylglycerol (IMTG) [10,27–30]. Another frequently used model in the literature to investigate the role of FFA in the onset of insulin resistance is the infusion of a lipid emulsion to increase the level of plasma fatty acids before and during a hyperinsulinemic euglycemic clamp. Elevating FFA levels by means of lipid infusion in humans, decreased insulin-

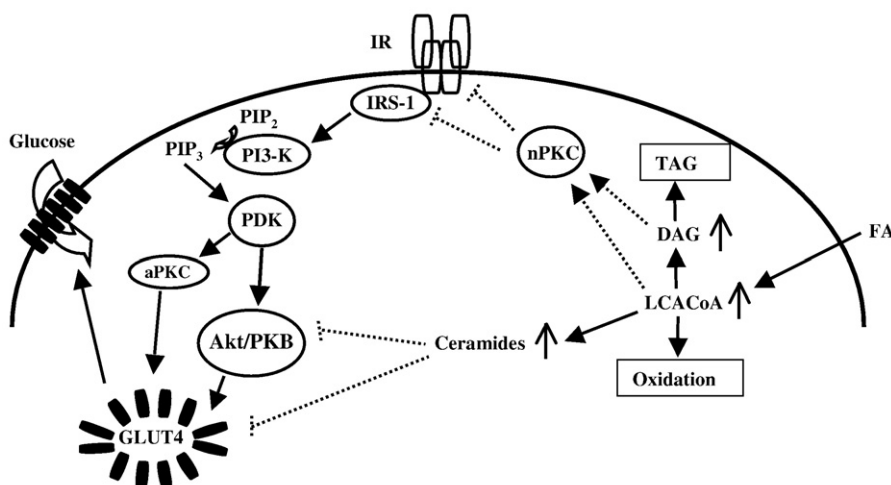


Fig. 1. Pathway of insulin-stimulated glucose uptake. Binding of insulin to the IR activates PI3K through IRS1. Downstream of PI3K, PDK mediates activation of Akt/PKB and aPKC. Activation of Akt and aPKC results in the translocation of GLUT4 vesicles from the cytosol to the membrane, facilitating glucose uptake. Also, fatty acids enter the muscle cell and are either oxidized or stored as TAG. Increased fatty acid delivery to the cell might lead to accumulation of LCACoAs, ceramides and DAG, which could interfere with normal insulin signalling by activation of nPKCs, serine/threonine phosphorylation of IR/IRS1, and impairing Akt phosphorylation ultimately reducing GLUT4 translocation. IR, insulin receptor; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3 kinase; PDK, phosphoinositide-dependent kinase; Akt/PKB, Protein kinase B; aPKC, atypical protein kinase C; LCACoA, long-chain acyl-CoA; DAG, diacylglycerol; nPKC, novel protein kinase.

stimulated glucose uptake in skeletal muscle, which provides evidence that FFA interfere with insulin action [22,31,32]. Interestingly, insulin resistance developed only 2–4 h after an acute increase in the plasma FFA concentration and it takes an equally long time to disappear after plasma FFA levels have returned to basal levels. From these observations, it can be concluded that the insulin desensitizing effect is not due to circulating fatty acids. It has therefore been suggested that fatty acids first need to accumulate in skeletal muscle before they can interfere with insulin signalling [33].

Indeed, ectopic storage of lipids as IMTG might play an important role in the onset of insulin resistance. This hypothesis is supported by experiments with lipodystrophic mice and humans. Lipodystrophy is a disorder characterized by the partial or complete loss of adipose tissue. Excess calorie ingestion in these mice or humans leads to high levels of ectopic lipid accumulation with decreased insulin sensitivity as a result. Interestingly, Gavrilova et al. [34] and Kim et al. [35] showed that this ectopic lipid deposition could be reduced by transplanting adipose tissue from wild-type mice into A-ZIP/F-1 mice, which have a severe form of lipodystrophic diabetes. Transplantation of wild-type fat reversed hyperglycemia, lowered insulin levels, and improved muscle insulin sensitivity, together with a reduction in ectopic fat storage. These studies suggest that not only lowering of ectopic fat depots but also adipose tissue secretory proteins may be important for normal glucose and lipid homeostasis. In that respect, treatment of lipodystrophic mice and humans with the anorexogenic adipocyte-derived hormone leptin [36,37] resulted in a remarkable improvement in insulin-stimulated liver and muscle glucose metabolism together with lowering fat stores [38].

Also, treatment of obese Zucker rats with Thiazolidinediones (TZD) support the hypothesis that muscular fat accumulation leads to insulin resistance. TZDs activate peroxisome proliferator γ (PPAR γ) receptors in adipocytes, and promote adipocyte differentiation, which results in the redistribution of fat from liver and muscle into adipocytes and as a consequence an overall improvement in insulin sensitivity [39]. However, recently it has been shown that treatment of type 2 diabetics with the PPAR γ agonist Pioglitazone may improve insulin sensitivity both by affecting serum adipokine concentrations and by reducing the intracellular triglyceride content of liver and skeletal muscle [40].

4. IMTG versus lipid intermediates

Although many studies associate IMTG storage with insulin resistance, the causality is not entirely validated. First of all, endurance training in obese humans improves skeletal muscle insulin resistance with little or no changes in IMTG concentrations [41,42]. Furthermore, Phillips et al. [43] have shown that endurance training in healthy volunteers progressively increased IMTG deposits, while simultaneously improving insulin sensitivity. In line with this, our group has recently shown that a short-term endurance training program increased IMTG content before insulin sensitivity was improved [44]. Another remarkable finding is that elite endurance trained athletes are amongst the most insulin-sensitive people and yet exhibit extremely high IMTG concentrations [45]. This metabolic paradox suggests that it is

unlikely that the IMTG pool is responsible for the reduced insulin action. Nowadays, this paradox is explained by the accumulation of lipid intermediates such as fatty acyl-CoA, ceramides and diacylglycerol, which might suppress insulin sensitivity [46] (Fig. 1).

4.1. Long-chain acyl-CoAs

Long-chain acyl-CoAs (LCACoAs) are the metabolically activated form of intracellular fatty acids produced by the action of the enzyme Acyl-CoA synthetase. LCACoAs are transported into the mitochondria for Beta-oxidation or used as an intermediate in the production of other lipid species. Increased uptake of FFAs or breakdown of IMTG stores could lead to high levels of intracellular LCACoAs. This form of lipid may influence energy metabolism acutely by changing substrate availability or by altering key enzyme activities via allosteric regulation. Increased muscular LCACoA levels have been associated with insulin resistance based on numerous animal and human studies in which the LCACoA levels are modulated by different dietary interventions or acutely via lipid infusion [27,47–49]. In addition, incubation of isolated rat soleus muscle strips with fatty acids reduced glucose uptake in a manner that correlated with the accumulation of LCACoA [50]. Moreover, interventions such as a single exercise bout or overnight fasting decreased the intramuscular LCACoA content in 3 week high-fat fed rats and increased insulin-stimulated glucose uptake into skeletal muscle [51]. Several mechanisms have been postulated that link LCACoAs with decreased insulin signalling. LCACoAs were shown to modulate enzyme activity of hexokinase in muscle and therefore directly regulate glucose influx. In liver, LCACoAs have been shown to modulate directly or indirectly the activity of key metabolic enzymes such as acyl-coA synthase, glucokinase, glucose-6-phosphatase, hormone sensitive lipase or AMP kinase [52]. Alternatively, LCACoAs might interfere with insulin signalling by activation of PKC isozymes [53,54]. However, despite the afore-mentioned evidence, LCACoA could also be a marker of increased muscular ceramide or DAG levels, which are associated with insulin resistance, as elevated LCACoA levels are often accompanied by elevated levels of the latter.

4.2. Ceramides

Ceramides can be generated from two fatty acids and serine via 4 enzymatic steps. They have a function as signalling molecule in several important physiological events including inhibition of cell division and stimulation of apoptosis. The synthesis of ceramides largely depends on the availability of long-chain saturated fatty acids, which together with serine are the rate limiting substrates in the *de novo* ceramide synthesis by serine palmitoyltransferase (SPT) [55]. SPT is specific for saturated FFAs, in contrast to incorporation of the second FFA in ceramides, which can originate from saturated or unsaturated FFAs [55]. Ceramides are also proposed to play a role in the development of insulin resistance since elevated ceramide levels have been shown in tissues of insulin-resistant animals [56], in skeletal muscle of lipid infused humans [57], and in skeletal muscle of obese insulin-resistant

humans [58]. Ceramides could accumulate in the muscle via hydrolysis of sphingomyelin, one of the four common phospholipids found in the lipid bilayer of the cell, catalysed by the enzyme sphingomyelinase [59], or via *de novo* synthesis from saturated fatty acids [60]. Tumor necrosis factor α (TNF α) has also been shown to stimulate sphingomyelinase and hence increase ceramide levels [61]. This might be important since an increased inflammatory state is also observed in obese individuals [62]. Ceramides might mediate their antagonistic effect on insulin signalling via inhibition of the phosphorylation of Akt [62,63], independently of changes in the ability of insulin to stimulate IRS-1 phosphorylation or PI3 kinase activation. Indeed, Hajduch et al. [64] showed that incubation of L6 muscle cells with the short-chain ceramide analogue C2-ceramide for 2 h resulted in a complete loss of insulin-stimulated glucose transport and glycogen synthesis, caused by a failure to activate Akt. Also incubation of C2C12 muscle cells with the saturated fatty acid palmitate leads to elevation of ceramide and inhibition of Akt and thereby reducing insulin signalling [63]. Moreover, blocking ceramide synthesis in palmitate-treated myotubes, restored Akt activation by insulin [65]. Taken together, there is evidence for a role of ceramides in the development of muscular insulin resistance. However a precise understanding of the molecular mechanism by which ceramides regulate insulin action is still elusive [62]. For a more extensive review about the role of ceramides in insulin resistance see Summers et al. [62].

4.3. DAG

DAG is another interesting candidate that might be responsible for lipid-induced insulin resistance. DAG levels have been found to be associated with insulin resistance after high-fat feeding in rats, in obese Zucker (*fa/fa*) rats and in denervated soleus muscle [66–71].

DAG can be derived from multiple sources (Fig. 2). First of all, breakdown of phospholipids by the enzyme phospholipase C (PLC) can generate DAG [72]. Secondly, DAG can be generated from phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PC). However, in case of lipid-induced insulin resistance a third principal source, the *de novo* synthesis of DAG by esterification of two LCACoAs to glycerol-3-phosphate, is probably the most important route. During the first acylation step catalyzed by glycerol-3-phosphate acyltransferase (GPAT), a fatty acyl-CoA is linked to the first (*sn*-1) position of the glycerol molecule, giving rise to lysophosphatidic acid (LPA). The second fatty acyl-CoA is attached to the second (*sn*-2) position of the glycerol backbone by the enzyme lysophosphatidate acyltransferase (LPAAT), forming a second precursor: phosphatidic acid (PA). The latter is subsequently transformed into DAG through the action of phosphatidic acid phosphohydrolase (PPH). TAG could then be formed by the action of diacylglycerol acyltransferase (DGAT) [73]. Alternatively, hydrolysis of TAG by the activity of lipases also results in increased DAG levels. Until recently, hydrolysis of TAG was believed to occur via hormone sensitive lipase (HSL). However, the observation that HSL deficient mice accumulate DAG [74], was the first indication that another myocellular lipase should be present for

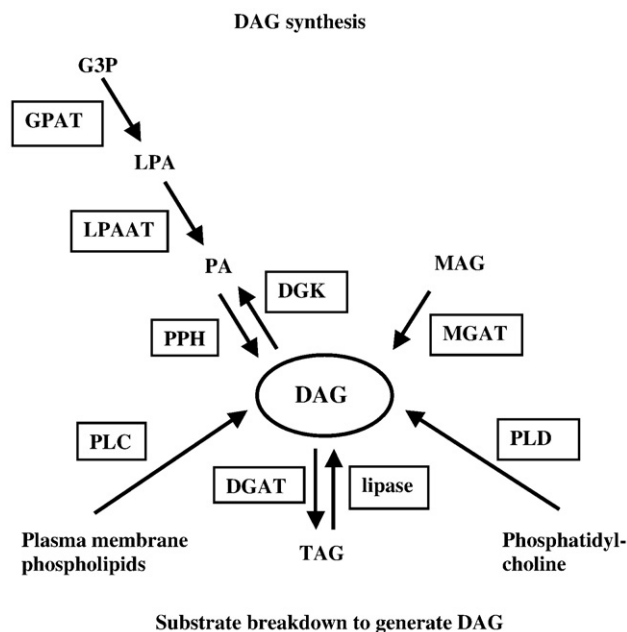


Fig. 2. Schematic representation of the synthesis of DAG. Upper part of the figure shows the synthesis of DAG from G3P or MAG. The lower part of the figure shows the generation of DAG by enzymatic breakdown of TAG, phospholipids, or PC. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; MAG, mono acylglycerol; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; PPH, phosphatidic acid phosphohydrolase; DGK, diacyl glycerolkinase; MGAT, acyl-coA: monoacylglycerol acyltransferase; PLC, phospholipase; PLD, phospholipase D; DGAT, diacylglycerol acyltransferase.

hydrolysis of IMTG. Recently, AdiposeTriGlycerideLipase (ATGL) [75–77] was discovered and this enzyme is now regarded as the main lipase responsible for hydrolysis of TAG to DAG. Indeed overexpression of ATGL resulted in massive increases in DAG levels [77] and might increase the risk to develop insulin resistance.

DAG is thus important as an intermediate in TAG metabolism, and it also occupies a central position in the biosynthesis of glycerophospholipids, which are the main components of cell membranes [73]. Furthermore, DAG is an important second messenger involved in intracellular signalling and thought to have detrimental effects on insulin signalling through its ability to activate PKC [14].

PKC is a family of structurally and functionally related proteins derived from multiple genes after alternative splicing [78,79]. PKC is activated in a response to a transient increase in DAG or after the exposure to exogenous tumour-promoting agents known as phorbol esters, which results in the translocation of the protein from the cytosol to the plasma membrane. A total of 12 isoforms of PKC have been cloned and characterized so far, which are classified into three different subfamilies [80]. Each isoform shows a different pattern of tissue distribution, substrate specificity and cofactor requirement. The classical PKC isoforms (cPKC α , β I, β II, γ) are calcium- and phospholipids-dependent and are activated by phosphatidylserine (PS), calcium and DAG (or phorbol ester). The novel PKCs (nPKC δ , ϵ , θ , η) are calcium-independent but phospholipids-dependent; they require PS and

DAG for activation. The atypical PKC isoforms (aPKC ζ , ι/λ) are calcium and phospholipid independent. Although the aPKCs are dependent on the presence of PS for their catalytic activity, these isoforms are not activated by calcium, DAG or phorbol esters.

During activation these kinases can translocate from the cytosol to the cell membrane and the extent to which PKC is found in membrane fractions is commonly used as a measure of its activation. As a consequence of prolonged activation, PKC can also be downregulated by proteolysis.

Activation of one or more PKC isoforms leads to a variety of biological responses, including changes in cell proliferation and differentiation, transmembrane ion transport, glucose and lipid metabolism, smooth muscle contraction and gene expression [81–83]. Very little is known about the functional specificity of different PKC isoforms. Animal studies have mainly implicated the nPKCs, PKC ϵ , and PKC θ in muscle insulin resistance [66,84,85] whereas activation of aPKCs can lead to insulin-mediated GLUT4 translocation to the plasma membrane, which enables glucose uptake in skeletal muscle [86].

Nowadays, DAG activation of the novel PKC isoforms is considered as a major determinant of lipid-induced insulin resistance.

One mechanism through which PKC could account for the insulin signalling defect is phosphorylation of the IR. It seems that serines on the β subunits of the IR kinase are an important target for PKC phosphorylation [87]. Serine phosphorylation, instead of the normal insulin-stimulated tyrosine phosphorylation, has been considered to be one of the mechanisms for inhibition of IR function [88]. Specifically, the structural mechanism appears to involve IRS protein dissociation from the insulin receptor by inducing conformational changes, thereby impeding access to tyrosine phosphorylation sites [89,90].

In the immediate downstream signalling events following IR stimulation, PKCs also phosphorylate and modulate the IRS proteins [91]. There are over 70 potential serine phosphorylation sites on IRS-1, and in general, serine phosphorylation seems to negatively regulate IRS signalling. Studies have demonstrated hyper-serine phosphorylation of IRS-1 on ser³⁰², ser³⁰⁷, ser⁶¹², and ser⁶³² in several insulin-resistant rodent models [92–95], as well as in lean insulin-resistant offspring of type 2 diabetic parents [96]. In vitro studies have shown that serine phosphorylation may lead to dissociation between the IR/IRS-1 and/or IRS-1/PI3 kinase, preventing PI3 kinase activity [97–100]. Ultimately, this IRS-1 inhibition will result in decreased insulin-stimulated glucose transport (GLUT 4) activity and glucose uptake.

5. A link between DAG and insulin resistance

In rodents, a number of studies have provided evidence for the association between aberrant activation of PKC and decreased insulin sensitivity, especially associated with increased lipid loading in insulin-responsive tissues. Alterations in specific isoforms of PKC were observed in skeletal muscle of high-fat fed rats. An increase in the proportion of membrane-localized PKC ϵ , PKC θ and PKC δ , which correlated with IMTG and DAG content was found in red skeletal muscle from high-fat fed rats, suggesting chronic activation, while the diminished total

amounts of PKC θ and PKC δ also indicated proteolysis [66]. In addition, a five-hour lipid infusion was also shown to increase the translocation of PKC θ from the cytosol to the membrane in skeletal muscle [85]. A study by Yu et al. [101] showed that infusion of a lipid emulsion in rats resulted in a three-fold increase in intracellular DAG mass, which was associated with PKC θ activation. Coinciding with the increased PKC θ activity, they observed a 30% reduction in insulin activation of IRS-1 tyrosine phosphorylation and approximately a 50% reduction in IRS-1 associated PI3 kinase activity. PKC activity, DAG content and PKC ϵ and PKC θ membrane localization were also increased in a study of obese Zucker rats [70]. Moreover, treatment of high-fat fed rats with an antilipolytic drug rosiglitazone, which reduced muscle DAG and IMTG levels, improved insulin sensitivity and reversed the diet-induced changes in nPKC expression [102]. Similarly, rapid reversal of insulin resistance in high-fat fed rats by an acute dietary intervention with one high-glucose low-fat meal also normalized PKC θ activation [103]. Furthermore, PKC θ knockout mice are protected from fat-induced insulin resistance in skeletal muscle. Kim et al. [92] showed that in contrast to wild-type mice, PKC θ knockout mice had no decreases in insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1-associated PI3 kinase activity after a five-hour lipid infusion.

In humans, both PKC θ protein content and activity were significantly increased in the rectus abdominus skeletal muscle from obese diabetic subjects compared to skeletal muscle from obese, normoglycemic controls, suggesting involvement of this isoform in type 2 diabetes [104]. Recently, the same group has shown that fatty acid induced muscle insulin resistance in normal volunteers was found to be associated with increased DAG mass and with PKC isoform $\beta 2$ and θ membrane translocation [32].

Taken together, these studies strongly support a link between IMTG, DAG accumulation, insulin resistance and activation of PKC, although the relative roles of the different PKC isoforms involved remain unclear.

6. Modulation of DAG

As reviewed above, substantial evidence links DAG accumulation, via activation of the nPKC isoforms, to the development of muscular insulin resistance. This makes DAG content an important target for therapeutic interventions.

As DAG is an intermediate in the synthesis of TAG from glycerol and fatty acids, its levels can be lowered either by improving the oxidation of cellular fatty acids by genetically modifying carnitine palmitoyl transferase 1 (CPT1) [105], or by accelerating the incorporation of fatty acids into TAG. The latter can be achieved by genetically modifying enzymes that are involved in the synthesis and hydrolysis of TAG, such as diacylglycerol acyltransferase (DGAT), glycerol-3-phosphate acyltransferase (GPAT), and stearoyl-CoA desaturase (SCD-1) [106–108].

DGAT catalyzes the final step in TAG synthesis by facilitating the linkage of *sn*-1,2-diacylglycerol with LCACoA. DGAT exists in two primary isoforms: DGAT1 and DGAT2 [108,109]. DGAT1 is ubiquitously expressed in human and mouse tissue, with the

highest expression levels in the small intestine and white adipose tissue [108] and it appears to have a role in fat absorption and energy homeostasis [110]. DGAT2 is primarily expressed in liver and white adipose tissue [109]. Revealing the function of DGAT2 has proven to be more difficult because homozygous DGAT2 knockout mice die shortly after birth due to severe lipopenia and impaired skin barrier function [111].

Since DGAT1 is present in all insulin-sensitive tissues, manipulation of DGAT1 activity could be an attractive target for manipulation of DAG content. It can be hypothesized that an increase in DGAT1 activity would increase the conversion of DAG into TAG, thereby lowering DAG content and improving insulin sensitivity. Conversely, ablation of DGAT1 could be expected to result in elevated DAG levels. Consistent with the role of DGAT1, mice lacking DGAT1, showed 30–40% decreased levels of TAG in adipose tissue and skeletal muscle [112]. In accordance with the decrease in adiposity, DGAT1^{-/-} mice have an improved whole body insulin sensitivity as shown by an increase in glucose infusion rate during hyperinsulinemic euglycemic clamp studies [112]. Insulin-stimulated glucose transport is increased in skeletal muscle and white adipose tissue of DGAT1 deficient mice, and the insulin-stimulated activity of key molecules in the insulin signalling pathway, such as PI3K and Akt is also increased in skeletal muscle of these mice [113]. Partial deletion of DGAT1 also appeared to improve insulin sensitivity, as evidenced by decreased blood glucose levels after intraperitoneal injections of insulin in inbred DGAT1-heterozygous (DGAT1^{+/-}) mice [114].

Although it is not surprising that the leanness in DGAT1 deficient mice leads to an improved insulin sensitivity, the precise mechanisms that contribute to the insulin sensitivity remain unresolved. It is hypothesized that DGAT1 deficiency in white adipose tissue alters its endocrine function [112,115], thereby promoting energy expenditure and sensitivity to insulin [116]. To test this hypothesis, white adipose tissue was transplanted from DGAT1 deficient mice into wild-type recipient mice and its effects on glucose disposal and the response to a high-fat diet was monitored. The recipient mice acquired partial obesity resistance, increased insulin-stimulated glucose disposal, and enhanced activation of the insulin signalling pathway [113,117]. These results strongly suggest that the altered expression and secretion of adipocyte-derived factors exert beneficial effects on energy and glucose metabolism. It therefore is of considerable interest to determine the adipocyte-derived factors that contribute to the overall improved insulin sensitivity in DGAT1 deficient mice. It appears that adipocytes that lack a functional DGAT1 enzyme have an increased leptin sensitivity, and DGAT1 deficient mice lose more weight than wild-type mice in response to subcutaneous leptin infusion [112]. Furthermore, expression of adiponectin, a white adipose tissue-derived hormone that improves insulin sensitivity and increases fatty acid oxidation, is also increased in DGAT1 deficient mice [118,119].

Although tissue TAG levels are reduced in DGAT deficient mice, levels of DAG and fatty acyl-coA, substrates of the DGAT reaction, are not elevated and even tended to be lower in skeletal muscle and liver [112]. These findings disagreed with the expectations that based on the function of DGAT1, DAG levels

would be increased, which would worsen the insulin sensitivity. Yen et al. [120] proposed that the absence of muscle DAG accumulation might be explained in part by the loss of acyl-CoA: monoacylglycerol acyltransferase (MGAT) activity mediated via DGAT1. This implies that DGAT1 could catalyze the sequential esterification of two fatty acyl moieties to convert monoacylglycerol to diacylglycerol and then to triacylglycerol. In other words, fatty acid uptake might be altered in DGAT^{-/-} mice. If this is indeed a function of DGAT in intact cells still requires further testing.

Contrary to the above mentioned findings, skeletal muscle-specific transgenic overexpression of DGAT1 in mice protected against fat-induced insulin resistance [121]. Myocellular overexpression of DGAT1 resulted in decreased DAG and ceramide levels and increased IMTG levels. The reduction in high-fat diet-induced insulin resistance could in part be attributed to attenuation of fat-induced activation of DAG-responsive PKCs. The level of membrane-bound PKC activity of all the DAG-responsive isoforms combined were lower in the high-fat fed DGAT overexpressing mice than in high-fat fed wild-type mice, but were comparable to the levels of normal chow-fed wild-type mice. Measurement of DAG-sensitive PKC isoforms β II, ϵ , and θ confirmed these results.

Moreover, a role for DGAT1 attenuating lipid-induced insulin resistance was shown in isolated muscles from whole body DGAT1 knockout mice, muscle-specific DGAT1 overexpressing mice and wild-type mice, which were incubated with a combination of palmitate and oleate [121]. Muscle from DGAT1 overexpressing mice showed enhanced insulin sensitivity compared to muscle from wild-type mice, whereas DGAT1 deficiency was associated with diminished skeletal muscle insulin sensitivity upon administration of fatty acids. The attenuation of fatty acid-induced insulin resistance in DGAT overexpressing mice was associated with decreased DAG and ceramide levels comparable with levels observed in control mice. In accordance, the decreased insulin sensitivity in soleus muscle of DGAT1 deficient mice correlated with increased accumulation of DAG mass and increased ceramide levels. These results are in contrast with the earlier mentioned results of Chen et al. [112], who showed decreased DAG levels in whole body DGAT1 knockout mice, and an improved overall insulin sensitivity, again suggesting that altered systemic factors (i.e. circulating adipokines) in these mice may have affected the phenotype observed in skeletal muscle [113].

Thus, it seems that the levels of DGAT1 in skeletal muscle indeed determine muscle insulin sensitivity and susceptibility to fatty acid-induced insulin resistance, as evidenced by decreased DAG and ceramide levels.

Improving the oxidation capacity of muscle cells can also modulate DAG levels. Indeed, Sebastián et al. [105] showed that incubation of L6E9 muscle cells overexpressing CPT1 with palmitate were protected against fatty acid-induced insulin resistance by inhibiting both the accumulation of lipid metabolites such as DAG and ceramides, and the activation of PKC θ and PKC ζ . In the absence of palmitate incubation CPT1 overexpression affected neither basal nor insulin-stimulated glucose metabolism. After a 16 h incubation with palmitate, insulin no

longer stimulated glucose uptake in control cells. This insensitivity correlated with the accumulation of TAG, DAG and ceramide. However, in CPT1 overexpressing cells insulin still stimulated glucose uptake after palmitate treatment to the same extent as in control cells without palmitate addition. This protection was associated with a reduction of palmitate incorporation into cellular lipids, and reduced levels of DAG and ceramides.

Another way to manipulate DAG may be via acute exercise and/or endurance training. Bruce et al. [41] showed that an 8 week endurance training program increased insulin sensitivity, accompanied by decreased levels of DAG. This decrease in DAG was not accompanied by changes in IMTG content. Schenk and Horowitz [122] tested the hypothesis that a single session of exercise in human subjects would protect against fatty acid-induced insulin resistance, when subjects received a 4 h lipid infusion. It was shown that one single bout of exercise was capable of markedly improving insulin sensitivity. This was accompanied by an increase in IMTG content, suggesting that a single bout of exercise may reduce the storage of fatty acids as metabolically unfavourable fatty acid metabolites. A possible mechanism for increased IMTG storage after exercise was the enhancement of the lipogenic capacity of skeletal muscle, as evidenced by increased protein expression of mGPAT, DGAT1, and SCD1. Ikeda et al. [123] also showed that a two week exercise training in mice increased the gene expression of DGAT1.

Next to exercise and genetic manipulation of genes involved in TAG metabolism, DAG may also be manipulated by dietary fatty acid composition. Studies in lipid-pretreated cells have shown that different types of fatty acids can lead to differences in the content of fatty acid intermediates [124,125]. Chavez et al. [124] showed that incubation of C2C12 myotubes with palmitate, but not oleate, inhibited insulin-stimulated glycogen synthesis accompanied by accumulation of DAG and ceramides. Incubation with longer chain saturated fatty acids also reduced insulin sensitivity and elevated DAG and ceramide levels, whereas incubation with medium chain fatty acids had no effect on insulin sensitivity or accumulation of DAG or ceramides. Similar results were obtained by Montell et al. [125] and Gaster et al. [126]. They showed that incubation of muscle cells with palmitate increased the incorporation of fatty acids into DAG whereas the unsaturated fatty acids were diverted towards storage in the TAG pool or present in the FFA pool [125].

An explanation for the effect of fatty acid type on the conversion towards TAG might be a different affinity of DGAT1 for the saturated versus the unsaturated fatty acids [125]. Coleman and Bell [127] showed that DGAT, isolated from fat cells, had a maximal activity when di-olein was used, whereas DGAT activity was minimal when di-palmitin was used as a substrate. Likewise, in L6 myotubes, DGAT also had a maximal activity for unsaturated acyl-CoAs, compared with saturated acyl-CoAs [128]. Moreover, Montell et al. [125] showed that increasing the concentration of oleate in a mixture of palmitate and oleate progressively declined the incorporation of palmitate into DAG, whereas the TAG concentration increased.

Taken together, these studies suggest that especially saturated fatty acids lead to the accumulation of DAG in muscle cells, whereas unsaturated fatty acids preferably stimulate the conversion into TAG. In that context it is interesting to note that it has been

shown that diets high in saturated fatty acids are more prone to result in insulin resistance compared to diets rich in unsaturated fatty acids in humans [129].

However, the number of studies that have addressed the effects of different fatty acid subtypes on insulin resistance *in vivo* in humans and animals is scarce. A possible way to examine this relationship is by means of the lipid infusion model. Studies using lipid emulsions to increase plasma fatty acid levels and muscular fatty acid intermediates in rats and humans showed an increase in DAG associated with increased PKC activity and decreased insulin sensitivity in rats and humans [32,101]. Both studies only observed an increase in the level of muscular DAG and not in intracellular ceramide or TAG concentrations, suggesting that these metabolites do not play a major role in mediating fatty acid-induced insulin resistance in skeletal muscle. This model could also be used to examine the effect of fatty acid subtype on lipid intermediate accumulation but until now studies have almost exclusively used intralipid, which is mainly composed of polyunsaturated fatty acids, as lipid emulsion. Therefore, it would be very interesting to study the effects of other lipid emulsions, with increased contribution of saturated fatty acids, on DAG accumulation, DAG type, PKC translocation and muscular insulin resistance.

7. Conclusion and future perspectives

In this review, we have summarized the effects of ectopic lipid accumulation on skeletal muscle insulin resistance. Among the many lipid metabolites that might contribute to the onset of insulin resistance, DAG has attracted particular interest. DAG is an intermediate in the synthesis of TAG from FFA and numerous studies have shown an association between DAG accumulation and the degree of insulin resistance.

If indeed the current sedentary life-style and the continuous access to energy-rich food is associated with high DAG levels, modulation of DAG might be an important therapeutic strategy to improve overall insulin sensitivity. It has been shown that DAG can be decreased by physical activity. Furthermore, dietary fatty acid composition has the potential to affect DAG content, although a limited number of publications are available. Therefore, more (human) research is needed to unravel the exact effect of fatty acid composition on DAG levels and insulin resistance.

An area that so far has been hardly addressed is the role that different subtypes of DAG may play in the development of insulin resistance. It can be suggested that the effect of fatty acid saturation and chain length on muscular insulin resistance as observed in muscle cells is not only due to an effect on DAG content, but also on DAG composition. So far, the analysis of different DAG species and their effects on PKC activity or translocation within the cell has been proven difficult, but is an area that certainly deserves further investigation.

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